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REVIEW

Functional aspects of protein flexibility

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Abstract Proteins are dynamic entities, and they possess an inherent flexibility that allows them to function through molecular interactions within the cell, among cells and even between organisms. Appreciation of the non-static nature of proteins is emerging, but to describe and incorporate this into an intuitive perception of protein function is challenging. Flexibility is of overwhelming importance for protein function, and the changes in protein structure during interactions with binding partners can be dramatic. The present review addresses protein flexibility, focusing on protein–ligand interactions. The thermodynamics involved are reviewed, and examples of structure-function studies involving experimentally determined flexibility descriptions are presented. While much remains to be understood about protein flexibility, it is clear that it is encoded within their amino acid sequence and should be viewed as an integral part of their structure.

Keywords Protein dynamics · Protein–ligand interactions · Protein flexibility · Flexible protein recognition model · Entropy · Intrinsically disordered proteins

Introduction

Many textbooks in the life sciences depict proteins as rigid building blocks, analogous to children's LEGO bricks, adding to an artificial static picture of proteins. Protein structures are, nevertheless, highly dynamic, and their biological functions depend intimately on this. The dynamics or motions in a protein allow its conformation to change and respond to the presence of other molecules and/or to variations in the environment. Biological and biochemical processes such as signal transduction, antigen recognition, protein transport and enzyme catalysis rely on this ability to change conformation or to *adapt* to change. The adaptability, or as we will refer to it here, the *flexibility* of a protein may result in either subtle changes as when a few amino acid side chains of an enzyme move to bind a small substrate, or in more dramatic changes as when the folding of certain proteins is facilitated by the presence of the appropriate ligand. The timescale of the conformational events that underlie protein flexibility spans 13 orders of magnitude (Fig. 1). The fastest events are librations and vibrations of covalent bonds and fast side chain rotations on the picosecond to nanosecond timescale. At the other extreme of the timescale, protein–ligand dissociation and protein (un)folding may happen with time constants of hours. A full description of the dynamics and motions in a protein requires the application of both experimental (primarily spectroscopic) techniques and molecular dynamics (MD) simulations. MD simulations have especially been powerful for the characterization of fast dynamics on the (sub-)nanosecond timescale [1, 2]. Recently, an MD simulation of ubiquitin in explicit solvent for as long as 1.2 μs has, however, been reported [3], which is very promising for future characterization of functional flexibility in proteins by MD.

That proteins are dynamic was realized from early studies of exchange of labile protons in proteins [4–9]. From these and later studies, it was also shown that the dynamics is not uniformly distributed throughout the protein and that highly dynamic sites are often involved in

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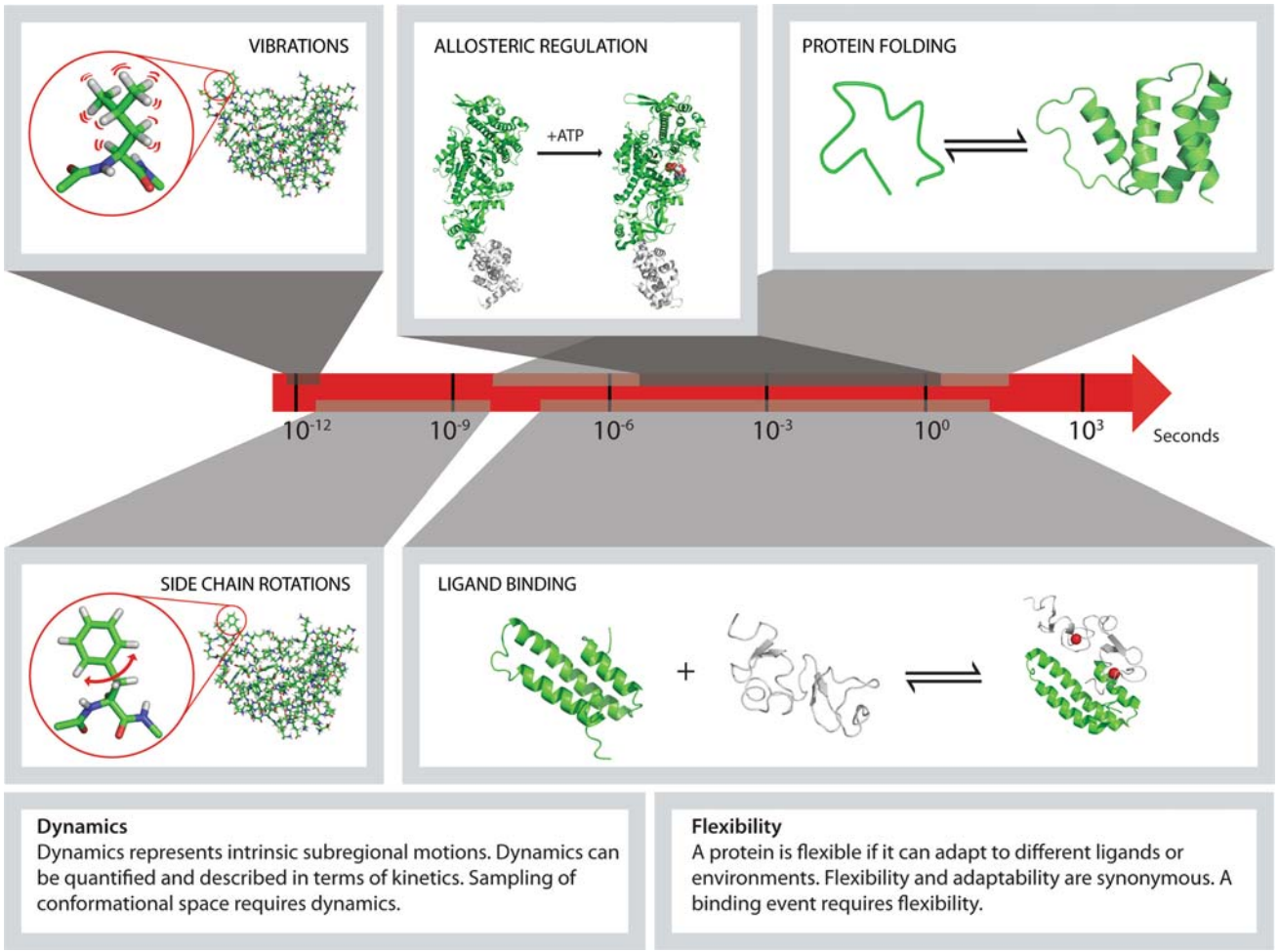

Fig. 1 Schematic illustration of conformational events in proteins. The motions that change a protein's conformation occur with time constants from nanoseconds to hours. The fastest motions, vibrations and librations around covalent bonds, result in atoms moving only a fraction of an Ångström. Ligand binding may involve only subtle motions like rearrangement of amino-acid residues in the binding site or larger movements over several Ångströms like domain reorientations as shown here for the binding of CR56 from LRP to RAP [123]. Similar movements may be observed for allosteric structural changes where binding of a ligand at one site of the protein changes the structure in another part. This is shown here for myosin V where binding and conversion of ATP to ADP in the upper domain (*green*) induces structural rearrangements not only around the nucleotide-binding site, but also in the distant lever arm domain (*grey*) [164, 165]. The ADP molecule is shown in *colored spheres* in the upper domain. Lastly, protein folding involves large movements of the whole protein chain as illustrated here for acyl-coenzyme A binding protein [166]. The fundamental terms dynamics and flexibility are defined at the bottom of the figure. Images of structures were generated in PyMol (DeLano Scientific)

protein–protein interactions [10, 11]. Indeed, limited proteolysis [12] has been used to demonstrate that although sites for a specific protease are available on the surface of a protein, cleavage does not necessarily take place, suggesting that availability is not the main determinant for protease activity. Rather, productive proteolysis depends on the flexibility and adaption of the peptide chain to the active site of the protease [13–15]. Additionally, an inverse relationship between protein stability and the biological function of both enzymes and protein hormones has been described, underscoring the fact that function necessitates flexibility [16, 17].

Here we use the term dynamics for intrinsic molecular motions. All proteins are dynamic, although with different amplitudes of the motions. We use the term flexibility for the ability of a protein to adapt its structure when it binds a ligand or in response to changes in the environment (Fig. 1). It is important to stress that proteins are flexible as a consequence of their dynamics, yet their dynamics do not automatically result in flexibility. This also implies that a very dynamic protein may not necessarily be very flexible—although this will often be the case.

The aim of this review is to provide an introduction to the current understanding of protein flexibility in a functional context. We have chosen to discuss mainly protein–ligand interactions, but it should be stressed that flexibility is also important for enzyme catalysis and protein (mis)folding. After an introduction to the thermodynamics

of ligand binding with a focus on the role of entropy, we describe several examples of how protein flexibility is involved in key biological processes. The examples have been chosen to illustrate how both timescale and amplitude for the motions that render proteins flexible may vary. Additionally, they emphasize the point that protein flexibility is an integral part of the structure/function relationship of a protein.

Thermodynamics of protein flexibility

To understand in detail the nature of protein flexibility and thus how it may influence the function of a protein, it is necessary to analyze the thermodynamics of the protein–ligand binding process. Every time a protein molecule changes its conformation, some interactions are broken and others are formed. The thermodynamics of the conformational change report on the types and numbers of interactions involved in the process. We will here briefly summarize the basic thermodynamics necessary to understand protein–ligand interactions and protein flexibility. As a simple example we will consider a protein P that binds a ligand L.

$$\mathrm{P} + \mathrm{L} \rightleftharpoons \mathrm{PL}$$

At equilibrium, the ratio between the concentrations of the free molecules P and L and of the complex PL is given by the equilibrium constant. For the reaction above, the equilibrium constant (the *association constant*), K_a, is:

$$K_a = \frac{[\mathrm{PL}]}{[\mathrm{P}][\mathrm{L}]},$$

Often the equilibrium constant for the dissociation reaction is reported. This is the *dissociation constant*, $K_d = 1/K_a$, and K_d is simply the free ligand concentration at which the protein is 50% saturated.

K_a may also be expressed in terms of a difference in the standard Gibbs free energy, ΔG°, between the free molecules and the complex:

$$\Delta G^\circ = -RT \ln K_a$$

$\Delta G^\circ < 0$ means that the energy of the complex is lower than that of the free molecules, i.e., the complex is energetically favored. In this case, formation of the protein–ligand complex PL is associated with release of energy, whereas dissociation of PL costs energy. Thus, at equilibrium each time one molecule of P associates with one molecule of L and release energy, a PL complex will dissociate using the same amount of energy.

ΔG° may be decomposed into changes in *enthalpy* (ΔH°) and in *entropy* (ΔS°)

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

The enthalpy change, ΔH°, is the change in the energy of all the interactions that stabilize the conformation of a system. For a protein these are covalent bonds, van der Waals' interactions, electrostatic interactions and hydrogen bonds. ΔH° for a process thus reports on the interactions that are lost or formed in the process. If $\Delta H^\circ < 0$, more stabilizing interactions are formed than broken in the process, and energy is released to the surroundings. The change in enthalpy will favor the reaction in the direction where $\Delta H^\circ < 0$. The entropy, S is a measure of the number of different ways a system can be arranged (that is the *disorder* of the system). The change in entropy, ΔS°, thus measures whether the system becomes more ordered ($\Delta S^\circ < 0$) or disordered ($\Delta S^\circ > 0$) in a process. If a reaction increases disorder, it is entropically favored.

The enthalpy and entropy contributions to the total free energy of a ligand-binding process almost invariably counteract each other. When a non-covalent interaction between a protein and a ligand is formed, it will decrease the enthalpy of the complex. Around the binding interface, the system will lose conformational freedom, and the entropy of the molecules will decrease. The more interactions that form, the more ordered the complex becomes. Indeed, a linear relationship between ΔH° and ΔS° has been found for a number of protein–ligand interactions (Fig. 2) [18]. Consequently, large values of ΔH° and $-T\Delta S^\circ$ for processes involving non-covalent interactions may result in only modest values of ΔG°. This is exemplified by the interaction between HIV-gp120 and CD4 for which $\Delta G^\circ = -50$ kJ mol^{-1} ($K_a = 5.8 \times 10^8$ M^{-1} at 298 K) [19]. This value is the result of very large and opposing ΔH° and $-T\Delta S^\circ$ values of -264 and 214 kJ mol^{-1}, respectively (lower left of Fig. 2) [19]. In this case, complex formation is driven by a favorable change in ΔH° and thus by the formation of intermolecular interactions. The formation of the complex at the same time induces order in the system. At the other extreme, the formation of a complex between porcine pepsin and pepsin inhibitor-3 is entropically driven ($\Delta H^\circ = 53$ kJ mol^{-1}; $-T\Delta S^\circ = -107$ kJ mol^{-1}), but still results in almost the same free energy difference: $\Delta G^\circ = -54$ kJ mol^{-1} ($K_a = 2.9 \times 10^9$ M^{-1} at 298 K) [20].

Entropy costs

As illustrated above, the binding of a ligand to a protein may result in an increase in entropy, a decrease in entropy or even no change in entropy. For protein–protein interactions, $-T\Delta S^\circ$ may vary considerably and is found in the range from ~ -100 to ~ 250 kJ mol^{-1} [21] (Fig. 2). Dissecting the entropy change for a process into specific

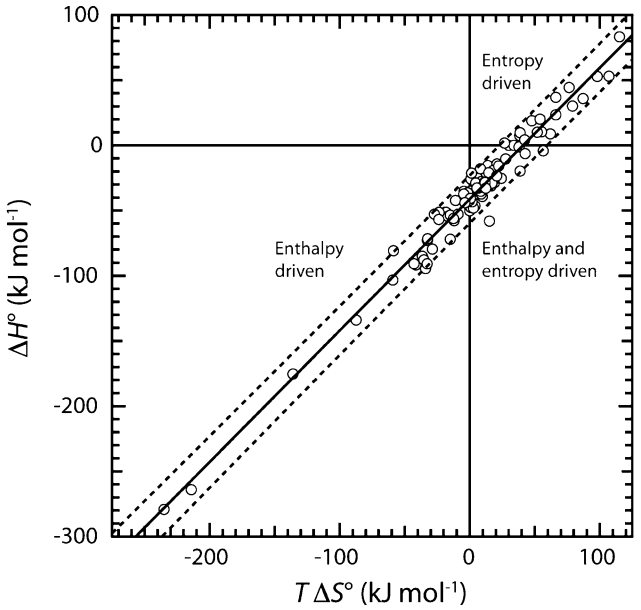

Fig. 2 Entropy–enthalpy compensation. Plot of $\Delta H°$ versus $T\Delta S°$ for the binding equilibria of 100 different protein–protein or protein–peptide interactions. The *solid line* represents the best fit of a *straight line* to the data (slope = 1.00 ± 0.04). The *dashed lines* indicate the 95% prediction interval from the fit. Data from [19–21, 167]

molecular events may provide valuable insight into the mechanism of the binding event and how the flexibility of the protein is involved in the binding process. The entropy change includes contributions from the protein, the ligand, and the surrounding solvent. The entropy changes for protein binding processes and protein conformational changes are complex. $\Delta S°$ may be divided into changes in translational and rotational entropy (ΔS_{tr}), conformational entropy (ΔS_{conf}) and solvent entropy (ΔS_{solv}) [22]. In order to appreciate how conformational changes and protein–ligand binding processes may be driven by protein dynamics and flexibility, it is essential to consider the relative importance of these three types of entropy.

The size of ΔS_{tr} depends on the type of the ligand and the nature of the stabilizing interactions [23], and determination of ΔS_{tr} from either experiments or MD simulations is accordingly complex. Reported values for ΔS_{tr} are typically in the order of 50 J mol^{-1} K^{-1} [23–25], corresponding to a change in translational and rotational entropy that destabilizes a protein–ligand interaction by approximately 15 kJ mol^{-1} at 298 K.

ΔS_{conf} depends on the conformational space available to the protein. ΔS_{conf} has been further dissected into contributions from backbone and side chains by calorimetric measurements in combination with MD simulations [26, 27]. It was found that for the backbone, the ΔS_{conf} associated with the folding of a protein is around 17 J mol^{-1} K^{-1} per residue depending on the type of amino acid [26]. This entropy change corresponds fairly well to a decrease in the number of configurations of the backbone ϕ and ψ dihedral angles from nine to one. The loss in conformational entropy of a side chain when the protein folds is on average 3.4 J mol^{-1} K^{-1} as long as the side chain stays solvent exposed. Subsequent burying of a solvent exposed side chain results in an additional entropy loss of 11 J mol^{-1} K^{-1} [27]. Using these values to roughly estimate the magnitude of ΔS_{conf} for a average protein–protein interaction where 1,600 Å^2 per subunit and 52 amino-acids are buried [28] results in a destabilizing contribution of 170 kJ mol^{-1} at 298 K.

The third and last contribution to the entropy term, ΔS_{solv}, results from the change in solvent exposed area on binding and is related directly to the *hydrophobic effect*. This entropic cost depends on the polarity of the surface. Hydration of a non-polar surface has a high entropic cost, whereas polar surfaces are generally considered to have a negligible contribution to ΔS_{solv} [26, 29, 30]. The entropic cost for the hydrophobic effect upon a change in non-polar surface area can be estimated from the following equation [30]:

$$\Delta S_{solv} = 1.43\,\mathrm{J\,mol^{-1}K^{-1}\AA^{-2}}\Delta A_{np}\ln(T/386)$$

where ΔA_{np} is the change in exposed non-polar surface area. Protein–protein interfaces have an average of 56% buried non-polar surface [28]. The entropy contribution ($-T\Delta S_{solv}$) from the hydrophobic effect to the stability of an average protein–protein complex at 298 K is thus in the order of −100 kJ/mol. From the discussion above it is evident that ΔS_{conf} and ΔS_{solv} dominate $\Delta S°$ for protein–protein interactions with opposing contributions of the same order of magnitude. Which contribution prevails depends, therefore, on the specific molecular structures involved in the process.

Entropy–entropy compensation

The binding of a ligand will inevitably result in ordering of atoms in the binding interface of the protein–ligand complex. From NMR relaxation experiments, it is possible to estimate residue-specific information about dynamics on the picosecond to nanosecond timescale [31, 32]. It has been demonstrated that this dynamics correlates with the free energy of binding, $\Delta G°$ and the change in conformational entropy, ΔS_{conf} [33, 34]. In many cases such measurements show that ΔS_{conf} of the protein is reduced when it binds a ligand [32]. This is what would be expected for the induced fit model of ligand binding (see below), where the binding results in structural rearrangement in the binding site to optimally accommodate the ligand [35]. However, in a number of cases it has been found that although the conformational entropy and thus dynamics decrease in the

binding site, redistribution of dynamics to other regions of the protein compensates for the entropy loss at the binding site, resulting in increased stabilization of the complex [36–43]. This effect has recently been termed *entropy–entropy compensation* [36]. The entropy–entropy compensation is most often partial, as for murine urinary protein I (MUP-I) and calmodulin [37–40]. However, in some cases, such as the binding of an effector peptide to the small GTP binding protein Cdc42Hs or the binding of D-galactose to arabinose binding protein, significant super-compensation has been reported [41, 43]. MD simulations of protein–ligand binding reactions have demonstrated both partial and super entropy–entropy compensations [42, 43]. NMR experiments and MD simulations show that especially increased side-chain dynamics is responsible for the entropy compensation [40, 42], but increased backbone dynamics has also been demonstrated [39, 43]. In a functional context, entropy–entropy compensations make sense especially for proteins that bind several different ligands such as MUP-I and calmodulin [37–40]. As binding sites may not be able to adopt optimal geometry for all ligands, non-covalent interactions between protein and ligand may not be strong enough to stabilize the complex by themselves. Increasing the conformational entropy outside the binding site is thus exploited by nature to provide the required energy for binding of many ligands to one protein.

Water

Water is intimately connected to the process of binding, because loosely bound water molecules at the protein surface are displaced from the binding site upon binding. A few water molecules may additionally contribute to both the stability and function of a protein. Many proteins have internal cavities with bound water molecules that stabilize the protein [44–46]. In contrast to the loosely bound molecules, these tightly bound water molecules [that is water molecules with well-defined interactions with the protein and long (>10 ns) residence times] may be directly involved in binding of the ligand through hydrogen bonds, thereby contributing to the energetics of the binding process. In two examples where tightly bound water molecules are observed (ConA/trimannoside complex and the HIV protease/inhibitor complex), they add as much as 64–72 kJ mol^{-1} to the stability of the protein–ligand complexes [47, 48]. Although tightly bound water plays a role for protein–ligand stability, the exact contribution is not easily determined [46–48].

Models for the mechanisms of protein binding

Protein–protein interfaces have generally been characterized as static portraits and have been described in relation to size, shape and complementarity [21, 28, 49–51]. Although important, the start and end points—i.e., the structures of the unbound protein and ligand, and of the protein–ligand complex—are insufficient to describe the whole binding process in details.

The first attempt to describe the actual process of binding was made by Emil Fischer who in 1894 suggested the *lock-and-key* model for enzyme-substrate interactions [52]. This model was based solely on shape and chemical complementarities between molecules without invoking changes in structures. Daniel Koshland later suggested the *induced-fit* mechanism based on the observation that a good substrate for an enzyme can induce a conformational change that activates the enzyme, whereas a poor substrate cannot facilitate this change [53]. In his lock-and-key model, Fischer assumed that the protein has a defined hollow space (or cavity) that a given molecule (ligand) fits perfectly into. In contrast to Fischer, Koshland included plasticity, or flexibility, into his model. The induced fit model was very early on extended to also explain the concept of *allostery*, which is when the binding of a ligand in one area of a protein affects the conformation in another area distant from the binding site [54, 55]. It was emphasized that an important condition for allostery is the presence of a significant fraction of residues with low structural stability (high flexibility).

A unifying mechanism of protein–ligand interactions

The induced-fit mechanism survived for more than 40 years before it was challenged especially by the influence from the new view of protein folding that emerged in the 1990s [56–58]. This introduced the idea that a protein moves on a multidimensional energy landscape rather than along a defined pathway when it folds. This energy landscape was illustrated as funnel shaped explaining parallel pathways as well as intermediates [59–61]. The state at the base of the funnel is the native state with the lowest energy. From the perspective that protein folding and protein recognition are similar phenomena in the sense that they share complexity and recognition, energy landscapes and funnel descriptions were also developed for protein–ligand interactions [62, 63]. Depending on the rate-limiting step in the binding process, different models emerged. These included the *pre-existing equilibrium* or *conformer selection* model [62, 64], and the *dynamic population shift* mechanism [65]. The foundation for these models is the unbound state, which exists as ensembles of conformations called conformational isomers or *conformers*. The conformers exist in equilibrium with variable energy barriers between them. If all energy barriers between the conformers are low, the sample can be described as a single Gaussian

distribution with only the average structure detectable. If the energy barrier between certain conformers or sub-populations of conformers is high, two or more distinct structures can be observed. The pre-existing equilibrium model states that there are high energy barriers between the conformers and that the ligands bind to a specific binding competent conformation in a process termed conformer selection [62]. When the energy barriers between different conformers are low, then binding of a ligand to a specific conformer leads to a shift in the equilibrium. This was suggested as the dynamic population shift model [65]. One example supporting this model is the nitrogen regulatory protein C (NtrC), which exchanges between an inactive and active conformation. When NtrC is phosphorylated, the population shifts towards the active conformation [66]. The presence of conformers with the structure of the bound state in an unbound protein ensemble is thus possible. This would imply that basal activities would be possible even in the absence of an activating ligand, which indeed has been described for G-protein coupled receptors [67, 68].

The different models were developed to explain specific data, but molecular dynamics simulations suggested that these models do not adequately explain the details of all protein–ligand binding processes [69]. In response to this, a unifying mechanism of protein–ligand interactions dubbed the *flexible protein recognition model* (FPRM) was recently put forward [69]. This model in essence explains the above-mentioned models as well as the induced fit. The model describes the binding process in three steps (Fig. 3), leading to three different complexes: (1) the encounter complex, (2) the recognition complex and (3) the final adapted complex.

The encounter complex

The first step is the formation of an encounter complex through diffusion and collision of the interacting molecules. In this high-energy complex, the interacting molecules still have the same structures (or ensembles of structures) as if free in solution, and the complex is mainly stabilized by long-range electrostatic interactions. The existence of encounter complexes in protein–protein interactions has been suggested from computer simulations [70] and later elegantly confirmed experimentally [71]. In these studies it became apparent that encounter complexes

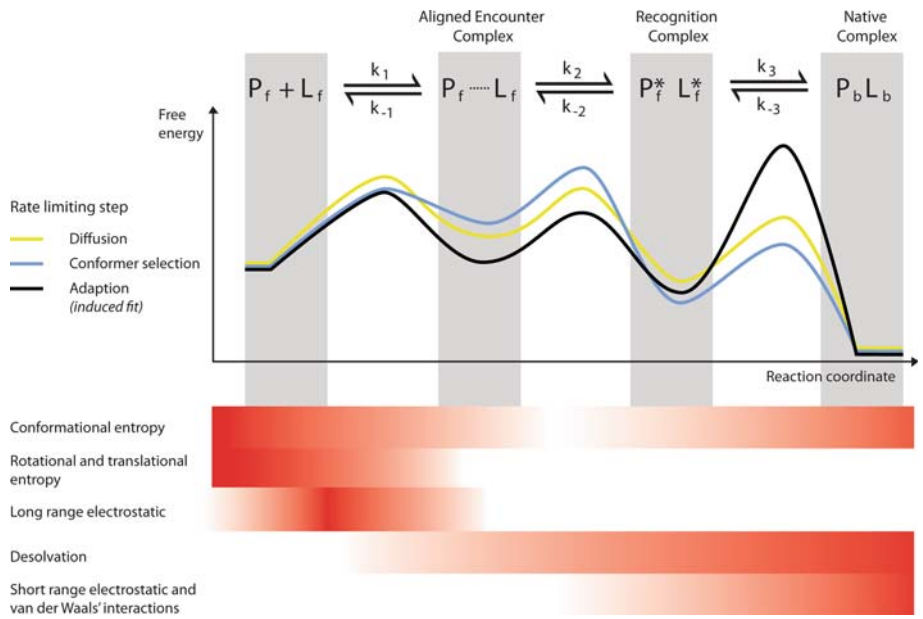

Fig. 3 The flexible protein recognition model (FPRM). The protein-binding process is divided into three steps. First the two molecules in the unbound ensembles, P_f and L_f, encounter by diffusion to form the encounter complex $[P_f \cdots L_f]$ driven mostly by long-range forces. Within the encounter complex environment, a gradual desolvation is initiated, and while still populating the free ensembles, the two molecules form the recognition complex via conformer selection $[P_f^* L_f^*]$ initiating a population shift. At this point short-range forces are in play. Within the recognition complex, induced fit and desolvation drive the formation of the final complex. Dependent on the barrier heights of the reactions, the protein-binding process may be controlled by diffusion (*yellow line*), by conformer selection (*blue line*) or by induced fit (large scale conformational changes) (*black line*). The forces that dominate the different steps during binding are shown schematically below. The intensity in color is relative between the different forces and not referring to any absolute magnitude. Formation of unaligned encounter complexes has been suggested, leading to a four-step binding reaction [168, 169]. Unaligned encounter complexes are not included in the FPRM described here. Figure modified from [69]

were directed (non-stochastic) and were located in or at the rim of the binding sites on each of the binding partners. The data obtained for the interaction of the phosphocarrier protein HPr with the N-terminal domain of enzyme I (EIN) suggested that the encounter complex interfaces are an order of magnitude smaller than the final binding interface [71]. When the rate-limiting step in a binding process is the formation of the encounter complex, the process is said to be diffusion controlled. A diffusion-controlled binding reaction is represented by the yellow line in Fig. 3.

The recognition complex

The second step is the formation of a recognition complex. In this high-energy complex, the interacting molecules have changed their structure to the most binding potent conformation found in the structural ensemble of the free molecules. Still only little desolvation and formation of short-range interactions have occurred. Conformer selection must occur within the aligned encounter complex and relies therefore on the adaptability or flexibility of the two binding partners within this partly desolvated environment. The blue line in Fig. 3 represents protein–ligand interactions that depend on the selection of conformers as the rate-limiting step in binding.

The final complex

In the final step, desolvation and short-range interactions drive the structural changes of the interacting molecules to give them their final bound conformations. This final step resembles the induced fit. The flexibility at this point of the reaction process must be paid for energetically, and therefore it must be assumed that the elimination of water needs to occur mainly in this step. For systems with large structural changes, the transition state energy is expectedly high, and most likely the rate-limiting step in binding. The black line in Fig. 3 represents these reactions.

Since the FPRM essentially describes the dependence of the reactions on the heights of the transition state barriers, it can be used to characterize the binding process regardless of whether it is diffusion controlled (formation of encounter complex), recognition controlled (conformer selection), induced fit controlled (final adaption) or controlled by a mixture of the three. MD simulations recently performed by Okazaki and Takada elegantly illustrate the usefulness of the FPRM. Their simulations showed a shift in the dominating control mechanism for different kinds of dominating forces and for different types of ligands [72]. Thus, small ligands had a preference for following the population shifts mechanism (recognition controlled), whereas larger ligands followed the induced fit mechanism (induced fit controlled) [72].

Crystallography and the study of protein flexibility

Protein flexibility imposes a number of challenges to the methods of study. Since the amplitudes and frequencies of the processes brought about by flexible chains cover a very broad interval, spectroscopy is and has been the method of choice. Several reviews have been dedicated to the descriptions of these methods as well as to their details and limitations [32, 73–76], and these will therefore not be dealt with here. Because X-ray crystallography does provide an important insight into flexibility from sampling of a number of structures and because B-factors are often referred to as flexibility reporters, we will briefly discuss the current status relating to these interpretations.

X-ray crystallography has proven extremely powerful with respect to the precise definition of atomic positions in three-dimensional space. However, the prerequisite of a crystallographic experiment is to have the molecule in the crystalline state, and the structure that can be derived from the diffraction pattern represents the conformer, which is common to all asymmetric units in the crystal. Should a loop region occupy more than one or two positions in space, it will not be represented by electron density peaks of sufficient magnitude for model building, nor will if be possible to conclude whether the lack of interpretable electron density is caused by the loop occupying several distinct minima or if it fluctuates freely. Crystallographic data are usually recorded at temperatures around 110 K, and the molecules are frozen in a conformation that is not necessarily a representative of the physiological state and that does not represent equilibrium. Temperatures below 200 K result in a low enthalpy structure with increased electrostatic intra- and inter-protein contacts [77].

The trajectory, velocity and frequency of the movement will also be unresolved. Still, X-ray crystallographic studies of enzymes trapped in different intermediates have provided valuable clues to the extent and function of protein dynamics in numerous systems [78]. These intermediates can represent steps in a catalytic cycle or a protein with and without its interaction partner (free and bound conformers) or simply one protein species crystallized under different conditions [79, 80]. Indirect evidence of protein flexibility has also come from the appearance of additives (especially Xe) in otherwise solvent inaccessible cavities in crystalline proteins [81, 82].

In the molecular model derived from crystallographic data, each atom is described by its center position, occupancy and an atomic displacement parameter, or B-factor. The B-factor reflects the degree of isotropic smearing of the electron density. A low B-factor means that the atom in question occupies the same position in all the molecules in the crystal and that the thermal vibrations are subtle. A high B-factor can arise from several phenomena: local

thermal vibrations (e.g., single side-chain movements), domain movements, occupation of more than one distinct position close in space, partial occupancy, etc. Not surprisingly, however, the core atoms of a protein molecule typically exhibit low B-factors, whereas the relative values of surface atoms are higher, demonstrating that the B-values contain information about local differences in dynamics.

It is possible to detect flexible regions from the B-factor distribution. However, the X-ray diffraction pattern arises from the molecule in the solid state, and crystal-packing interactions can introduce artifacts, which further complicate the deconvolution of the displacement values. Recent results show that if crystal-packing effects are taken into account, the B-factor correlates with dynamics and flexibility [83]. Furthermore, B-factors correlate to some extent with order parameters obtained from NMR relaxation studies and simulations [15, 84, 85].

Flexibility and function

In the remaining part of this review, we will describe a number of protein systems where flexibility is a key player in relation to function. We will describe both specific systems for which a certain point can be made and groups of proteins for which flexibility is a general trait relating to function. Whenever possible, we will link to the FPRM.

Small amplitude motions in binding site flexibility

The first example concerns small amplitude movements and their relation to substrate and inhibitor binding in acetylcholine esterase (AChE). Forty-seven different crystal structures of *Torpedo californica* AChE from three different space-groups and representing different protein–ligand complexes were compared [86]. The analysis was focused on the side-chain $\chi1/\chi2$ dihedral angles determining the orientation of a subset of aromatic amino acid residues at or near the substrate recognizing gorge and the active site. Two of the side chains (F330 and W279) existed in three and six different conformers, respectively. F330 acts as gatekeeper or bottleneck in the tunnel that leads to the catalytic site. It recognizes the substrate (or inhibitor) via π–π stacking or π-cation interactions and exists in either two or three distinct conformers depending on the ligand. Contrary to a 20-ns MD calculation, which predicted side-chain positions in favored regions only, some of the experimentally determined conformers occupied disfavored regions in a $\chi1/\chi2$ plot. The prevalence of F330 for the disfavored region turned out to be caused by the binding of a crystallization agent, polyethylene glycol 200 (PEG200). Hence, the side chain adopts a conformation not encountered in the unbound state, and thus the binding of PEG200 involves induced fit.

The other flexible side chain (W279) constitutes part of the substrate binding pocket and is found in six different conformations depending on the ligand [86]. This remarkable adaptability reflects low amplitude flexibility and a fast conformational sampling, and was confirmed by MD calculations, which corresponded very well with the experimental data [86]. Thus, MD calculations can reveal the number and distribution of allowed side-chain conformations independent of ligand interactions, even when crystal-packing interactions are taken into account. Apart from this being of potential interest in the field of drug design, it demonstrates that in this case, the induced fit model does not fully satisfy the observations. This can be seen as an example of conformer selection in which the interacting molecules sample several conformations within the aligned encounter complex. For this particular protein, the energy profile of the binding process may change depending on the ligand of choice (jump from blue to black reaction path, Fig. 3).

Large amplitude motions in enzyme activation

The second example demonstrates large amplitude movements in enzyme activation. The streptococcal pyrogenic exotoxin B (SpeB) is a cysteine protease that is secreted as an inactive zymogen [87]. As with most proteases, activation involves a proteolytic digestion, which releases a pro-domain from the active enzyme. In this particular example, inhibition by the pro-domain is indirect as its presence induces a conformational state of SpeB in which two residues in the active site, a tryptophan (W214) and the catalytic histidine (H195), adopt rotamer conformations that render catalysis impossible [87]. What make this system particularly spectacular are the very large amplitude movements. Two SpeB structures have been solved: (1) the inactive zymogen (proenzyme) with the 112 amino acid residues long pro-domain present [87] and (2) the proteolytically activated protein (253 amino acid residues) in solution [88]. Figure 4 shows the two states of the protein.

Removal of the pro-domain induces a large intramolecular rearrangement. A loop (the latency loop) moves more than 25 Å from one pole of the protein to the opposite pole, whereby it releases certain steric constraints in the active site. Another loop (the switch loop), which contains the catalytic histidine, is then liberated to move away from the substrate-binding site, and the active conformation is attained. Interestingly, the configuration of the active site amino acid residues in the active conformation is not arranged in the classical papain-like cysteine protease manner, indicating the need for further rearrangements upon substrate binding. The latency loop and a loop

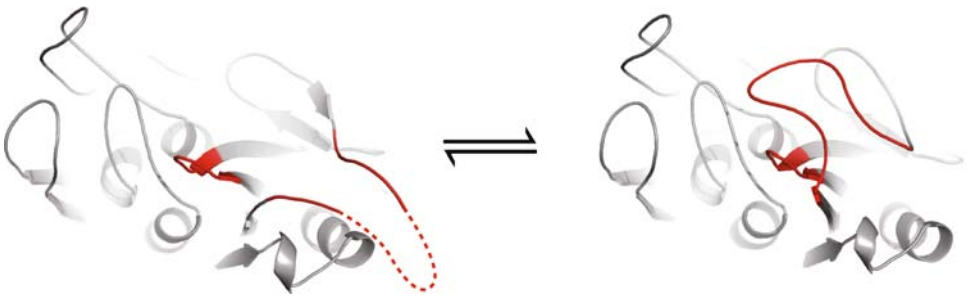

Fig. 4 Large amplitude movements in enzyme activation. Two experimentally determined structures of a cysteine protease from *Streptococcus pyogenes* with the long latency and short switch loops highlighted in *red*. The *dotted hand-drawn lines* connect the backbone where electron density did not support model building. *Left* the crystal structure of the zymogen (1DKI, shown without the pro-domain for clarity) and *right* the NMR solution structure of the proteolytically activated monomer (2JTC). The latency loop moves >25 Å upon removal of the pro-domain and the protein folds up into an activated form

containing an active site residue (Trp214) in a non-active conformation are highly dynamic compared to the rest of the molecule. Although crystallography cannot measure time-dependent movements, the large amplitude component of this system probably falls into the category of slow motions. Since the active state of the enzyme is not present in any of the structures, the primary enzyme-substrate encounter most likely has an induced fit contribution with large rearrangements facilitating catalysis.

Conformer selection and induced fit in antigen recognition

The last example from crystallographic studies comes from antibody–antigen interactions and demonstrates a conformer selection and induced fit scenario. The IgE antibody Spe7 raised against a 2,4-dinitrophenyl hapten exhibited the ability to bind to several small molecule haptens as well as to a protein ligand [79]. Some of these ligands are similar in molecular structure to the original antigen, and they bind in a similar fashion, representing examples of common molecular mimicry. However, additional high-affinity (nM–μM range) ligands with different physicochemical properties were also found. The structural basis of this highly specific cross-binding property was investigated by X-ray crystallography and binding kinetics [79].

Several structural conformers of the Spe7 antibody were seen in crystals grown under different conditions and with different ligands present. Two of these, referred to as Ab^1 and Ab^2, are the unligated protein and differ substantially in the third heavy chain (H3) and third light chain (L3) loops in the antigen-binding region. In the absence of ligands, experimental data suggested that Ab^1 and Ab^2 exist in equilibrium where Ab^1 occupies ~78% of the population [79]. These two conformations exchange relatively slowly with a half-life of ~9 ms. Only Ab^2 binds the different haptens, resulting in a protein–ligand complex with the antibody in a third conformation (Ab^3); see Fig. 5 for the full mechanism. Ab^1 does not bind the ligand mainly because of a clash of aromatic groups, which hinder isomerization in the presence of ligand. Fluorescence kinetic data revealed a fast, an intermediary and a slow step in the binding process. The rate of the fast step was positively correlated with ligand concentration, the second was negatively correlated, and the slow step was independent of ligand concentration. Thus, the fast phase is dominated by the initial ligand binding to Ab^2, the intermediate phase is dominated by the equilibrium between free Ab^1 and Ab^2, and the slow phase is dominated by the induced fit (Ab^2-ligand → Ab^3-ligand) (Fig. 5). The Ab^2-ligand complex is the $[P_fL_f]$ state in the FPRM model, and in this case, the rate-limiting step is the last one, between $[P_f{*}L_f{*}]$ and P_bL_b (Ab^3-ligand). The Spe7 system provides an interesting combination of promiscuity and high specificity at the same time. Promiscuity by low specificity has been described for several other systems. One example is the lipid transfer protein that binds a broad range of hydrophobic metabolites with low affinity and modest conformational changes [89–91].

The binding of ligands to AChE, SpeB and Spe7 are examples of processes where the binding is limited by the last two energy barriers in the FPRM. Indeed, most protein–ligand binding processes can be regarded as a combination of conformer selection and induced fit.

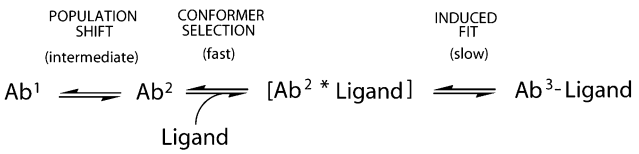

Fig. 5 Binding of ligands to the Spe7 antibody. Two conformers, Ab^1 and Ab^2, are in equilibrium, and only the Ab^2 conformer binds the ligand. The complex undergoes induced fit resulting in the stable Ab^3-ligand complex. The relative rates of the individual reactions are indicated

Compared to conformational changes, diffusion in aqueous solvents is intrinsically fast. Consequently, diffusion controlled binding processes usually involve few structural changes as, for instance, in the homo-dimerization of superoxide dismutase [92]. Therefore, diffusion-controlled binding is rare.

Intrinsic disorder and flexibility

A large part of the eukaryotic genome encodes proteins or parts of proteins with intrinsic disorder [93–95]. Instead of having an ordered fold, these intrinsically disordered proteins (IDPs) are dynamic ensembles of inter-converting conformers, with atom positions varying significantly over time [96–98]. The length of the disordered regions may range from only a few residues providing hinge functions [99, 100] to entire proteins of considerable sizes [98, 101]. Methods to predict the existence of disorder in proteins have been developed primarily with the scope of avoiding these sequences in structure/function studies [102, 103]. As the interest in the function of IDPs has increased, these predicting tools have turned out to be very important for the characterization of IDPs [104]. Several extensive reviews have described the intriguing characteristics of these proteins in great detail [97, 105, 106].

The question is whether the extreme disorder in IDPs also gives rise to extreme flexibility. There are several data to suggest that these proteins indeed are truly very flexible. Firstly, already within the dynamic ensemble of the IDP a pre-organization of local, fluctuating structures exists [107]. These structures have been described as central for recognition of the ligand [97, 108–110] and have been referred to as molecular recognition features (MoRFs) [109]. For recognition and function, these MoRFs are initially protected within a semi-condensed state of the IDP and structural rearrangements or partial unfolding must therefore occur prior to binding. These events are bound to depend on flexibility. Secondly, many of the IDPs are promiscuous with several interaction partners and fold to defined structures upon binding [106, 111, 112]. A single IDP may thus assume several different structures dependent on the ligand [113]. This adaption must certainly reflect an ultimate high degree of flexibility and not only extraordinary dynamics. This has been termed flexibility-driven poly-reactivity [97].

The diversity in bound states for one single protein with retained high specificity for all partners has, however, the consequence of inferring low affinity [94, 111, 114]. The low (μM) affinity is a prerequisite for the functions of many IDPs, which are often coupled to tightly controlled and transient processes such as signal transduction [115], cell-cycle regulation [107], cell-division [116] and growth [93]. The low affinity is on the other hand also a consequence of paying back a large entropy penalty going from the highly disordered state to the more ordered bound state [117–119]. To compensate the loss in conformational entropy, the majority of IDPs folds upon binding and gain a large enthalpy contribution from extended interaction interfaces [96, 106, 120, 121]. For a few IDPs the gain in enthalpy has also been linked to homo-dimerisation of the IDP subsequent to target interaction [122]. Another mechanism to lower the entropy loss is by assuming a modestly condensed shape, which in some cases can be obtained through dimerisation of the IDP itself [107].

Flexible linkers and termini

The presence of flexible linkers in otherwise ordered proteins have direct impact on function. Typically theses linkers are found in modular proteins such as large membrane-bound receptors [123] or in soluble modular proteins even as small as calmodulin [100, 124]. The sequences, dynamics or flexibility of these linkers, however short they may be, classify many of them as belonging to the IDP regime, and they are therefore discussed in this section. The linkers may be a short flexible hinge as in calmodulin [100] or long and disordered as in polypyrimidine tract-binding protein (PTB) with two RNA recognition motifs (RRMs) [125]. The two modules of each of these systems tumble and behave largely independently of each other, so the systems have a high degree of dynamics where the linkers allow for effective sampling of conformational space. As soon as one module binds to the target, the linker is stabilized, and the structure of the linkers adapts to accommodate the ligand. This suggests that the flexibility of the system may play a role also in the recognition event [126]. Hinge regions (or flex points) such as linkers thus impose flexibility and thereby facilitate variable orientations and increased binding diversity and may allow for multiple binding partners. Consequently, identification of flexible hinges is of great interest for modeling protein–ligand interactions, and several approaches to predict hinges in proteins from sequence and structure are available [127–129].

Another interesting feature is the presence of extended, disordered termini [130, 131]. These termini have been assigned many different roles. One is to capture targets by a fly-casting mechanism [132], in which a spatial search by the dynamic and flexible terminus is suggested to increase interaction speed that will lead to longer lifetimes of the resulting complex. For ion channels another function of the extended, disordered termini has been put forward. In several studies the length of the intracellular terminus was shown to affect the rate of channel inactivation and the flexibility of the chain was coupled to this effect [133].

The mechanism was suggested very early as the ball-and-chain model [134] and shown experimentally for other systems as well [133, 135]. Just recently, the ball-and-chain mechanism was linked to IDPs [109].

For IDPs, flexibility results in large amplitude motions, the frequencies of which are essentially unknown presently. The backbone of many IDPs have been shown to be highly dynamic on the nanosecond to picosecond timescale [107, 136], but the frequency with which the large amplitude change is occurring when going from the flexible unbound state to the ordered bound state is largely uncharacterized. Since the lifetime of the low affinity complexes formed by IDPs can be estimated to be in the order of $\sim$10 s (assuming $k_{on} = 10^5$ s^{-1} M^{-1}, and $K_d = 1$ μM), the frequencies may be classified to be in the slow range.

Within the framework of the present review, it is interesting to discuss at what point in the binding process of IDP flexibility is most effective and productive. Given the background of the universal binding mechanism outlined above [69], this suggests that the IDP initially binds to the target protein in the disordered form, possibly through the MoRFs [97, 109]. These MoRFs would not necessarily be important for the function per se, but may lead to an increased effective concentration of the IDP close to the functional site. One example of an IDP with a single MoRF is the suppressor of Mec 1 lethality, Sml1. It has an α-helical MoRF that recognizes ribonucleotide reductase 1, RNR1. When this MoRF was destabilized by mutagenesis, lower affinity of Sml1 for RNR1 was measured [107, 137]. Nevertheless, a small nine-residue peptide that is not part of the MoRF, but constitutes a sequence located C-terminal to it, was capable of fully inhibiting RNR1 by itself [137]. This suggests that the inhibitory function resides outside the MoRF. Thus, the binding process of an IDP strongly depends on flexibility both in order to present the MoRF (during encounter and recognition) and for the protein function, i.e., inhibition as for Sml1, to form the fully adapted complex through an induced fit process. Thus, presentation of the MoRF from a moderately condensed state of the IPD does involve specific flexibility or perhaps unfolding. Since flexibility at some degree is important during all steps of a binding process, the binding process of an IDP probably requires larger amplitudes than globular proteins, especially in the first stages of the binding process.

Proline *cis–trans* switches

Regulation of cellular processes, such as ion gating [138], signaling [139] and ligand recognition [140], are in some instances driven by conformational switches linked to *cis–trans* isomerisation of the peptide-bond preceding proline residues. Often these prolines are located in disordered and loop regions [141, 142]. While the process of *cis–trans* isomerisation is silent in many biochemical assays, it is readily detected by NMR spectroscopy because of the different chemical shifts of the two states [143].

The intrinsic low energy gap between the *cis* and *trans* conformations of the peptide bond preceding a proline results in a higher population of the *cis* conformation of these bonds compared to non-Pro peptide bonds [143]. Recently, there has been a significant increase in the number of reports suggesting that protein structures with proline switches have evolved to accelerate the isomerization process. This acceleration may be catalyzed by the binding of ligand [138] or by interactions with enzymes catalyzing the process, the so-called peptidyl-prolyl *cis–trans* isomerases (PPIases) or in combined events [142]. It has been suggested that the actual proline to isomerize may not be available for the enzyme until a conformational change or a chemical modification like phosphorylation is brought about. The latter is suggested to be the case for the PPIase Pin1 [144]. Thus, as a result of flexibility, particular interactions may in this way be timed [139, 144]. Uncatalyzed proline *cis–trans* isomerization (i.e., without ligand binding or enzymatic access) occurs in the folding peptide with rate constants in the range of 0.01–1 s^{-1} (half-time 100 s at 0°C) [145, 146], which may be even slower with a half time of several minutes when it occurs in the scaffold of a fully folded protein. In the native state of proteins, catalyzed proline *cis–trans* isomeri usually occurs on the millisecond timescale and may result in large amplitude structural changes, which may be accelerated up to 1,000-fold by PPIases [147, 148].

One of the most intriguing examples of the effect of *cis–trans* proline isomerization is the opening and gating mechanism of 5-hydroxytryptamine type 3 receptor (5-HT3) [138]. When a neurotransmitter molecule is bound to the extracellular domain, a *trans* to *cis* isomerization of a proline distantly located within a loop linking two transmembrane helices (Pro8*) is suggested to occur. A set of non-natural analogs that had different preferences for either the *cis* or the *trans* conformation were substituted for Pro8*. Elegantly, it was shown that opening and closing of the gate was intimately linked to the isomerization process, leading to a large amplitude conformational change [138].

Protein phosphorylation

The human genome codes for 20,000–25,000 proteins, but this is far from the true diversity in the organism [149]. Proteins are modified at many different levels, such as phosphorylations [150], lipidations [151], glycosylations [152] as well as proteolytic maturation [153, 154] or the

final degradation of the protein at the end of its path [155]. All these modifications are dependent on protein–protein interactions and must rely on both the enzyme to bind the protein target and the protein to make available the site prone for modification or cleavage. This process is inevitably dependent on flexibility of either interaction partner.

Phosphorylation of proteins has often been related to disordered regions [156] and to ordered regions in which phosphorylation may lead to disorder [157]. Many IDPs exert regulatory functions with phosphorylation as an intrinsic ingredient [116, 107]. The complex between Cdk4 and its inhibitor Sic1 was recently described by NMR spectroscopy, and it was suggested to be the first example of an IDP whose binding is devoid of a disorder-to-order transition [158]. The complex was termed a truly disordered complex [158]. Multiple phosphoepitopes on Sic1 were shown to bind to the core Cdk4-binding site in a dynamic equilibrium between many different bound conformers. Other phosphorylated residues interacting with suboptimal binding sites further stabilized binding. Since phosphorylation of several residues is required for binding, there is a tight regulation of activation. Apparently, the complex between Sic1 and Cdk4 appears to be less flexible although it is highly dynamic. As the complex is only marginally stable ($\sim$4 kJ mol^{-1}), it is only transiently formed and does not result in a folded complex [158]. In this system, dynamics are important for the phosphorylation and therefore the recognition process. In this case, flexibility required for formation of a long-lived complex, would be an obstacle rather than an advantage.

The effect of phosphorylation in ordered proteins may be seen as an allosteric site regulation process, for which the chemical modification not only changes the chemical environment, but which also leads to conformational switching. This suggests that single domain proteins also contain allosteric mechanisms as shown elegantly for NtrC [66], where phosphorylation results in conformational changes in the receiver domain. The experimental work suggested that upon phosphorylation, the pre-existing equilibrium was changed. In principle, the activation barrier for this equilibrium can be manipulated. For NtrC and CheY, a certain population of the active conformer was observed even in the absence of phosphorylation [67].

Discussion and conclusions

The purpose of the examples and thermodynamic platform presented in this review is to demonstrate that in order to appreciate and understand protein function entirely, dynamics and flexibility must be considered as a part of a protein structure. Flexibility as a structural aspect or an extra structural dimension is undoubtedly encoded within the amino acid sequence of a protein, just like secondary, tertiary and quaternary structures are.

Detection of flexible regions, or flex sites, within a particular structure is not an easy task. During recent years, technical advancements—mainly within the fields of spectroscopy and molecular dynamics—have made it possible to monitor the dynamics and functional flexibility of protein molecules, which in turn has precipitated an increased focus on these studies. Flexible regions have been deduced and characterized by NMR relaxation experiments. However, until recently, dynamics on the nanosecond to microsecond timescale have been inaccessible to experimental methods (cf. Fig. 1). This has been a major limitation since, as outlined in the present review; some motions correlated to functional protein flexibility lie in that frequency regime. Recent exciting results on measurements of residual dipolar couplings suggest that a method for accessing this regime may now finally be available [159].

From the discussions in the present paper, it is clear that the underlying mechanisms of functional flexibility in proteins do not differ much from the mechanisms of protein folding. Indeed, folding and binding of several IDPs are linked processes. The distinction between flexibility and folding is therefore not sharp. Several proteins are linked to disease through misfolding, and this misfolding may be a direct consequence of flexibility in their native states. Several human diseases and in particular neurodegenerative diseases are associated with the formation of insoluble protein inclusions—most often in the form of protein fibrils [160]. Such conformational changes require unfolding of globular proteins or partly folding of intrinsically disordered proteins [161, 162].

The flexibility-related movements within protein molecules can be massive, as described for IDPs, and they can be extremely subtle as for example by aromatic side-chain rotation as described for AChE. Furthermore, movements may be correlated. Thus, large amplitude flexibility may be correlated to low frequency and small amplitude movements as has been suggested [163]. However, much remains to be understood about how these correlated motions are connected and how they are triggered. Answering these questions will be one of the primary challenges of the coming years.

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